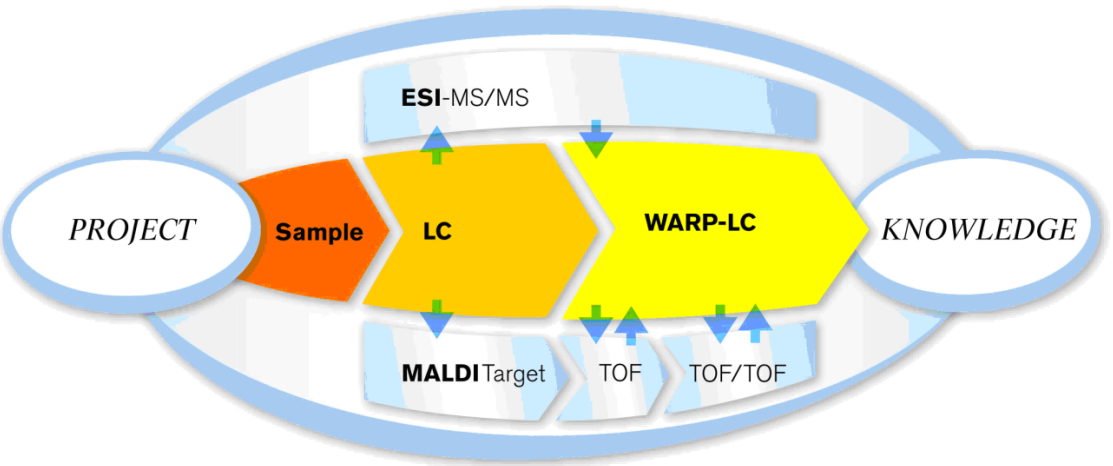
Application Tutorial

BRUKER

SILE Quantitation Using ESI Data



Revision 1 (November 2011)

Legal and Regulatory Notices

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Introductory remarks

Related software or higher:

ProteinScape 3.0 with WARP-LC 1.3

DataAnalysis 4.0

Mascot 2.3

This tutorial focuses on quantitation experiments using non-isobaric and isobaric labeling chemistry and Bruker mass spectrometers equipped with an ESI ion source.

1 Introduction

1.1 General

In **S**table **I**sotope **L**abeling **E**xperiments (SILE), quantitative information about proteins in complex mixtures is obtained. The current protein and peptide label chemistries are based on isotopomers of the labeling reagent. For example, ICPL, iTRAQ, SILAC, ICAT, and ^{18}O labeling can be used in the workflow described in this tutorial. Typically two states of a proteome or a protein/peptide mixture are compared in SILE. If more than two mixtures are compared in a single LC-MS/MS experiment it is called multiplex SILE.

The ESI workflow starts with LC-MS/MS analysis of the mixture of differentially labeled peptides, originating from different protein samples. The MS raw data are processed by DataAnalysis via a script to find compounds, and the exported peaklists are imported in ProteinScape. Here, the database search is started, and the peptide ratios are calculated by WARP-LC acting in the background. Protein ratios are calculated by ProteinScape.

- For non-isobaric label (e.g. ICPL or SILAC), the quantitative information is read out from the MS data. MS/MS can be used to identify solely those peptides that are regulated. DataAnalysis creates extracted ion chromatograms for the parent m/z of the identified peptides, integrates them and determines the area or intensities for the ratio calculation.

- For isobaric label (e.g. iTRAQ) the quantitative information is unraveled only in the MS/MS spectrum by the reporter ions of the different forms of the label (see Figure 1-1). Their intensities are used for ratio calculation.

Current label chemistries provide technical quantitation errors of less than 10 - 20 %, which is a lot better than the typical biological variation in these experiments.

SILE workflows can be performed interactively or fully automatically. Figure 1-2 gives an overview of possible starting points for quantitation. The different settings, which control the automatic execution of the workflow, are summarized in chapter 1.9.

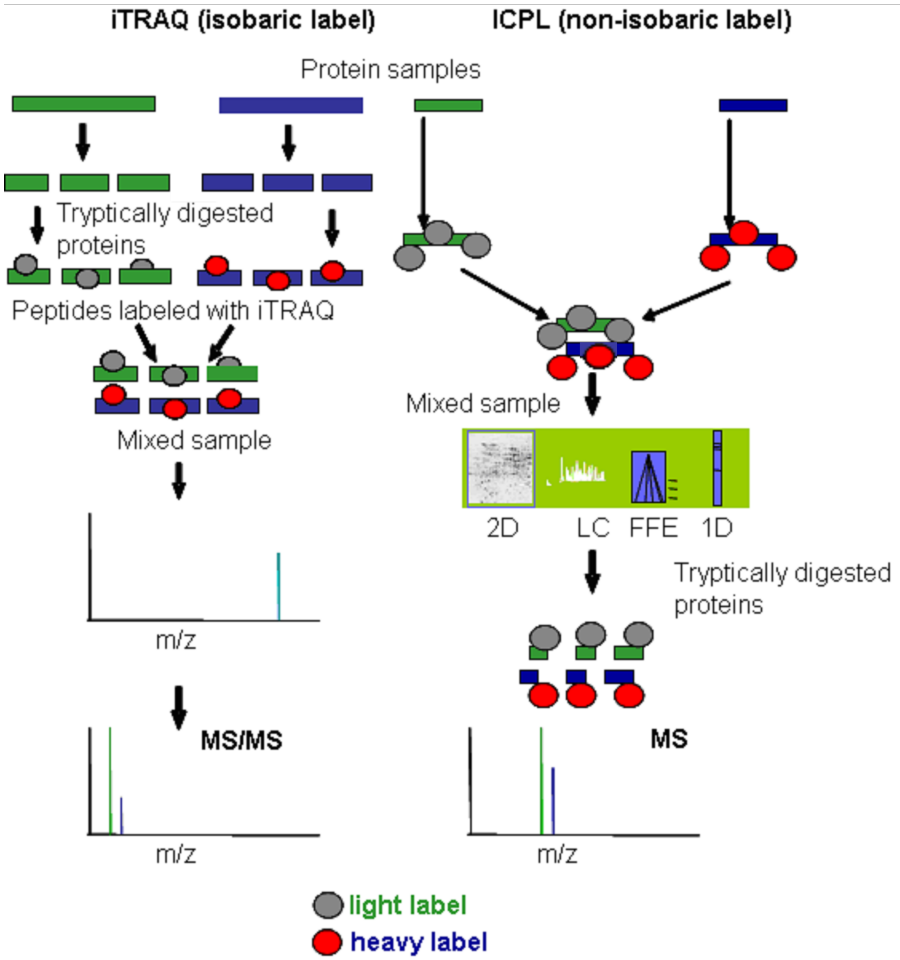

Figure 1-1 **Typical workflows for common label chemistries.: iTRAQ peptides are labeled post-digest, and quantitation is performed based on reporter ions in MS/MS spectra. ICPL permits protein pre-fractionation because labeling is done prior to protein digests.**

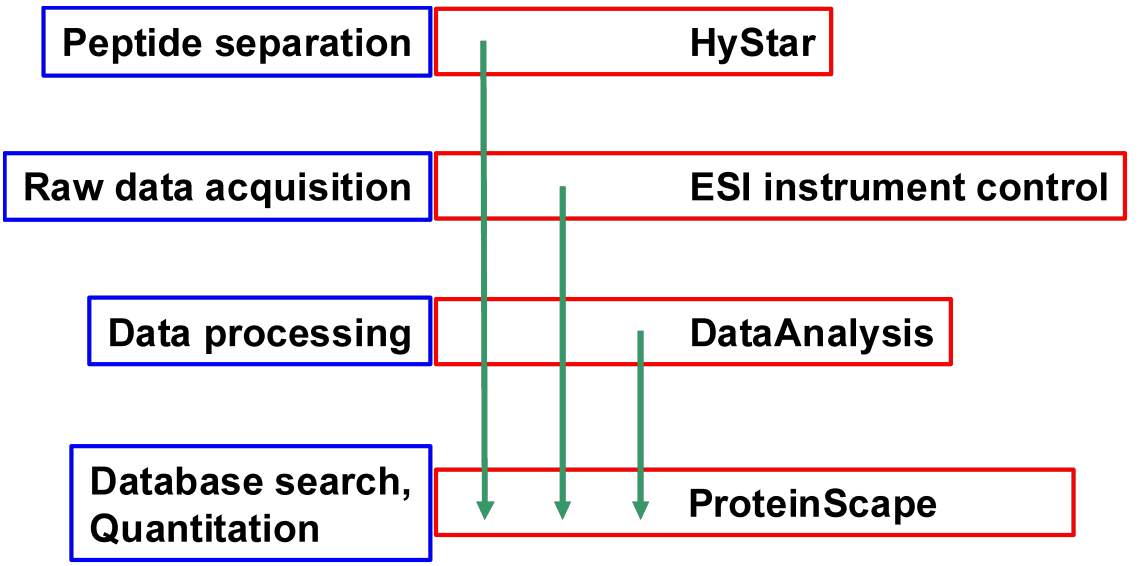

Figure 1-2 **Starting points for the quantitation workflow with ProteinScape as central element.**

1.2 Tutorial Data

The tutorial data `HCT_ICPL_Mix.d` are provided on the tutorial data DVD. They need to be copied to the computer used for the analysis, e.g to `D:\Data\Tutorial Data`.

Note For using the tutorial data ProteinScape 3.0, DataAnalysis 4.0, and WARP-LC 1.3 are required.

1.3 Method Overview

Default methods for SILE workflows are installed during the installation of WARP-LC. The installed methods are summarized in Table 1-1 and Table 1-2. They can be used for SILE experiments with ICPL or iTRAQ in combination with the specified mass spectrometer. The default methods need to be adapted for the user's experiment.

Table 1-1 Default methods for a non-isobaric SILE experiment with ICPL and Bruker ESI mass spectrometer.

Procedure	Software	Method name		Default location
Processing	Data Analysis	DA_4.0_IonTrap_ICPL_Default.m DA_4.0_maXis_ICPL_Default.m		D:\Methods\Processing\WARP_LC
Database search	Protein Scape	IonTrap_ICPL2plex_allOrg_GluC		PS Server
Quantitation	WARP-LC	WLC_1.3_IonTrap_ICPL2plex_Default	WarpLC Method	D:\Methods\WarpLCMethods

Table 1-2 Default methods for an isobaric SILE experiment with iTRAQ and Bruker ESI mass spectrometer.

Procedure	Software	Method name		Default location
Processing	Data Analysis	DA_4.0_maXis_iTRAQ_Default.m		D:\Methods\Processing\WARP_LC
Database search	Protein Scape	maXis_ iTRAQ4plex_allOrg maXis_ iTRAQ8plex_allOrg		PS Server
Quantitation	WARP-LC	WLC_ 1.3_ maXis_ iTRAQ4plex_Default WLC_1.3_maXis_ iTRAQ8plex_Default	WarpLC Method	D:\Methods\WarpLCMethods

1.4 HyStar

In HyStar a Sample Table is created. Use the **General** tab for setting injection details and data storage location. Use the **Method** tab for setting methods. For details please refer to the *HyStar User Manual*.

1.5 MS Instrument Control

The MS methods can be opened and edited in the respective instrument Control software. They need to be optimized for each ESI instrument type.

For **non-isobaric SILE** experiments, it is recommended to make use of the SILE option, which you find in the Advanced Auto MS(n) parameters of the instrument Control software. An example is shown in Figure 1-3. The parameters are explained in Table 1-3.

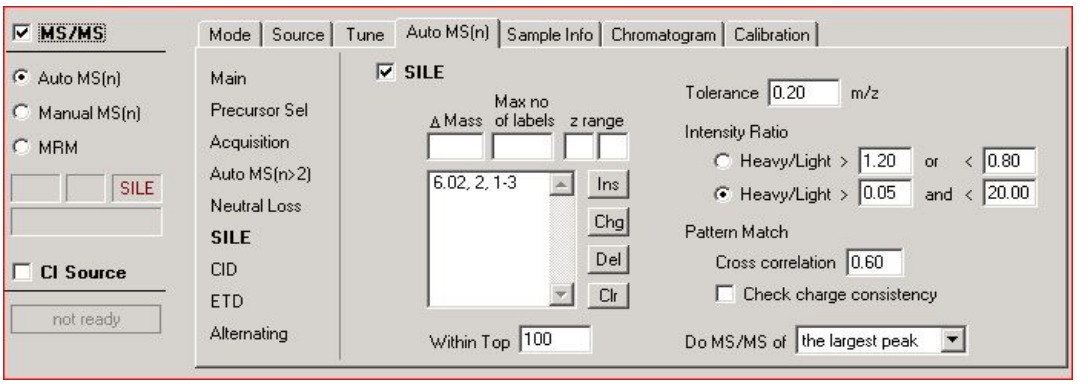

Figure 1-3 **SILE parameter tab in the IonTrap Control software showing parameters for an LC-MS/MS experiment with ICPL**

Table 1-3 Method parameters of the instrument control software for a non-isobaric SILE workflow.

Parameter	Explanation/ Recommendation
SILE	Check box for SILE precursor selection. Check it for non-isobaric SILE experiments.
Δ Mass	Mass difference between the heavy and light partner. Several mass differences can be specified for multiplex experiments.
Max no of labels	Maximum number of labels expected in a single peptide.
z range	Expected charge range for the labeled peptides.
Within Top	Specifies the number of peaks which are searched for SILE pairs. This value depends on the complexity of the sample.
Tolerance	Mass difference tolerance between the SILE partners. This value depends on the mass accuracy of the measurement.
Intensity Ratio	Defines which SILE pairs are of interest for MS/MS measurements. Use the Heavy/Light option greater x or smaller y if you are interested in strongly regulated SILE pairs.
Pattern Match	The cross correlation factor determines how good an observed isotopic pattern needs to fit to a theoretical pattern. The cross correlation factor for perfectly fitting isotopic pattern is equal to 1.0. It is lower for less matching peaks. A commonly used value for the required cross correlation factor is 0.6. Check charge consistency: if this option is checked the charge states of the SILE partners are checked before the pair is accepted. However, the charge determination of the less intense partner might be risky.
Do MS/MS of...	Choose which partner of the SILE pair shall be measured in an MS/MS experiment: the more intense, the heavy, the light, or both partners.

1.6 DataAnalysis

1.6.1 DataAnalysis Method

In DataAnalysis open the LC-MS/MS data file, select an appropriate DataAnalysis method via Method | Open, save the analysis, and run the script (Figure 1-4) via Method | Run.

Note For the non-isobaric workflow, it is essential to save the method with the data file, since adequate parameters are required for creating the EICs. However, this command is included in the script.

The DataAnalysis methods, which are provided with in the installation (chapter 1.3), contain:

1. Parameters optimized for finding compounds.

Some of them are instrument specific like those for mass annotation. In the following sections, important parameters for SILE experiments are found:

- **Find | AutoMS(n)** (compound finding)
- **Charge Deconvolution | Peptides/Small Mol.**
- **Mass List**
- **Export**

Note For the isobaric workflow it is recommended not to deconvolute MS/MS spectra in the range of the reporter ions, e.g. to start deconvolution at about 125 for iTRAQ.

2. An automation script (Figure 1-4): Details are described in chapter 1.9.
3. Parameters for creating EICs, which is required for the non-isobaric workflow:

- **Find | Chromatogram, MS(n)**
- **Process**

Note For the non-isobaric workflow the checkboxes in the Process tab need to be checked.

```
Option Explicit

Analysis.ProcessAutoMSn

Analysis.Compounds.Clear

Analysis.Save

Form.Close
```

Figure 1-4 DataAnalysis method script for the SILE workflow.

1.6.2 File Formats

For the non-isobaric usecase, the Bruker XML format is required.

For the isobaric usecase, other formats like MGF can be used as well.

1.7 WARP-LC

WARP-LC methods can be selected upon starting the **Quantitation** in ProteinScape. The methods provided with the WARP-LC installation are listed in chapter 1.3. They differ in the SILE labeling strategy and charge states (see below). The WARP-LC Method Editor organizes the method parameters on four. For editing them, the methods are opened in WARP-LC upon activating **Edit**.

1.7.1 Workflow Tab

On the Workflow tab, activate the LC-ESI SILE option for the workflows described in this tutorial.

1.7.2 SILE Chemistry Tab

On the SILE Chemistry tab, select the labeling strategy in the upper table: isobaric (Figure 1-5) or non-isobaric (Figure 1-6).

In the Biological Experiment section, define the **Biological States** which refer to the individual labeled samples, e.g. treated vs. untreated.

Each Biological State can have one or more chemical (or metabolical) **Labels**. They can then be combined in the “Labels assigned to Biological States” section.

In the Ratio Name section, the **Reported Ratios** are defined via the Numerator and Denominator columns. The Ratio Names need to be unique. The quantitative information of the proteins will be reported in the ProteinScape Protein Browser for all regulation ratios which have been defined.

In case of isobaric labeling strategy purity correction values can be specified. The **Purity Correction Values** dialog (Figure 1-7) is displayed upon activating the Purity Correction Values button. In the dialog all reporter ions which have been defined previously are shown. The purity correction values are supplied by the vendor of the labeling chemistry and are batch specific. They are considered in the calculation of the regulation ratios.

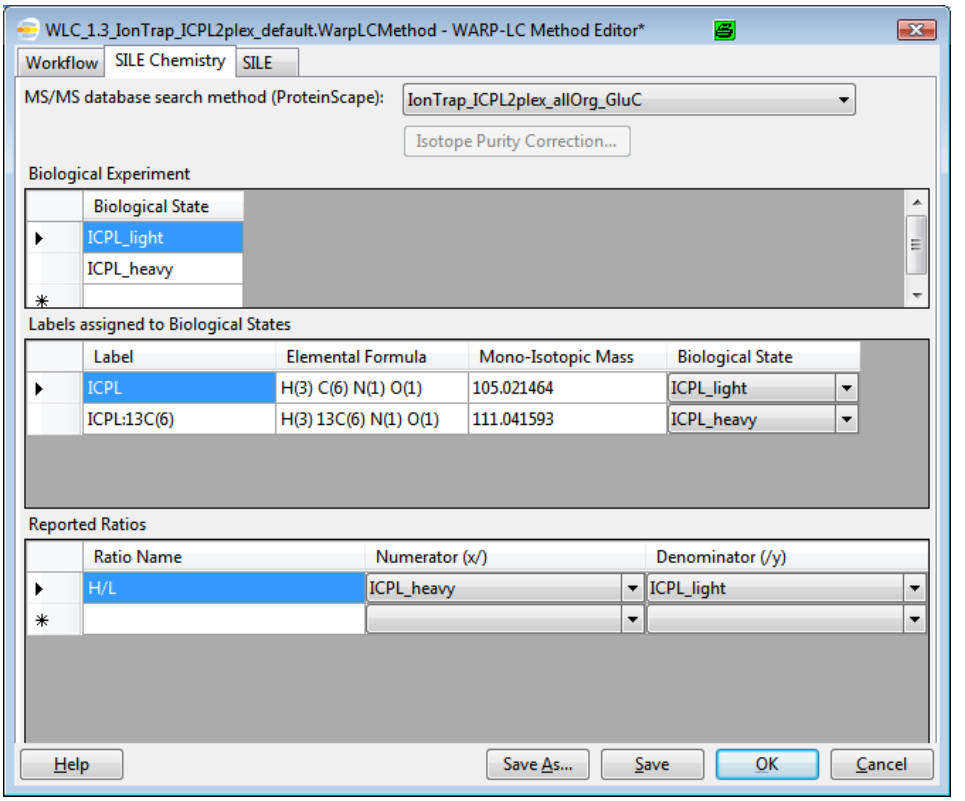

Figure 1-5 SILE Chemistry tab of the WARP-LC Method Editor for a non-isobaric modification.

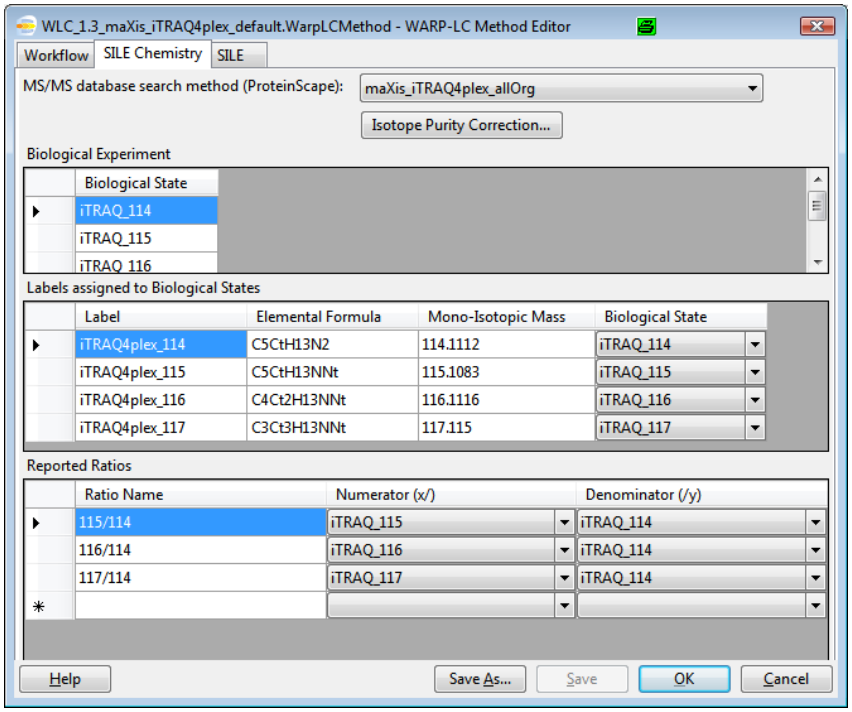

Figure 1-6 **SILE Chemistry tab of the WARP-LC Method Editor for an isobaric multiplex experiment.**

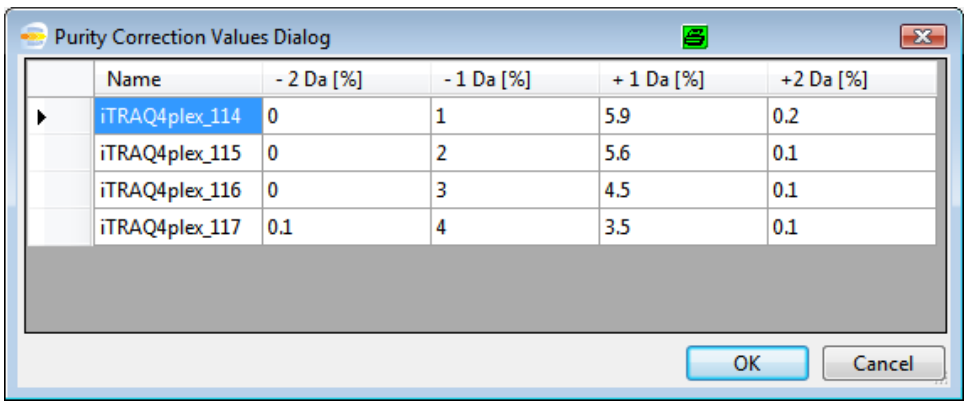

Figure 1-7 **Purity correction values for isobaric labelling experiments.**

1.7.3 SILE Tab

Parameters on the SILE tab (Figure 1-8) influence the calculation of the regulation ratios.

- For an isobaric labeling strategy only the Mass Tolerance parameter is available. It reflects the mass accuracy of the spectrometer in the reporter ion region.
- For the non-isobaric labeling workflow, more parameters can be set. They are explained in Table 1-4.

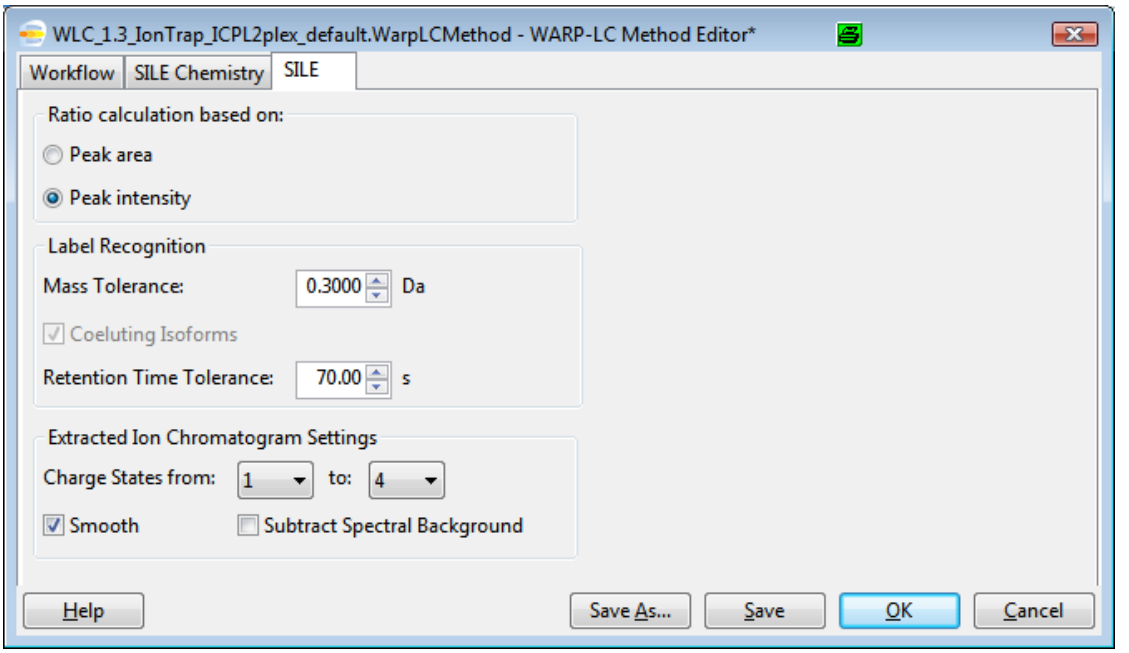

Figure 1-8 **SILE tab of the WARP-LC Method Editor for non-isobaric labelling. Except for the parameter in the MS/MS Peak Selection group box, all parameters influence the ratio calculation from compounds which are derived from extracted ion chromatograms in DataAnalysis.**

Table 1-4 Parameters on the SILE tab of the WARP-LC Method Editor for the non-isobaric workflow.

Parameter	Explanation/ Recommendation
Base Quantity for Regulation	"Area" or "Intensity" can be used in a non-isobaric SILE experiment. If less intensive chromatographic peaks are included in the quantitative analysis, "Intensity" is a more robust measure. For an isobaric experiment only "Intensity" is available.
Mass Tolerance (also available for isobaric labeling)	In case of a non-isobaric SILE experiment this parameter corresponds to the width in chromatographic trace definition in Edit | Chromatograms of DataAnalysis. In case of an isobaric SILE experiment this parameter is used to select the correct MS/MS reporter ion peak and reflects the mass accuracy of the instrument in the reporter ion region.
Retention Time Tolerance	Considers the retention time differences between the heavy and light partner of a regulation ratio. For co-eluting isotopomers of a label, the retention time difference should be in the range of the full width half maximum of a chromatographic peak.
Charge States	Charge states which are considered in an extracted ion chromatogram traces calculation in DataAnalysis.
Smooth	When smoothing is turned ON, the extracted ion chromatogram is smoothed before compounds are calculated from the extracted ion chromatogram. The method parameters for smoothing, which are set in Methods | Parameters | Process in DataAnalysis are used. Good results are achieved using the Savitzky Golay algorithm with 2 points smoothing width.
Subtract Spectral Background	When spectral background subtraction is turned ON, the spectral background that originates from the solvent used is subtracted from the measured data. This allows for detecting additional compounds having lower intensities than the solvent. As the intensity and area of these compounds might still be disturbed, this option does not necessarily improve the quality of the quantitation result. Parameters for the spectral background subtraction can be set via Methods | Parameters | Process in DataAnalysis.

1.7.4 Data Processing Tab

On the Data Processing tab the ProteinScape method, which is used for the database search, is selected.

1.8 ProteinScape

1.8.1 Database Search Method

In ProteinScape, start with the search methods, which come with the server installation. Figure 1-9 shows the search parameters of method `IonTrap_ICPL2plex_allOrg_GluC`, which cover the peptide identification. Figure 1-11 shows the assessment parameters for the same method. A useful assessment parameter for SILE quantitation is the radio button for selecting, which peptides are shown in the Protein & Peptide search result main table. Here, it is recommended to select “all compounds (SILE quantitation)”.

For database searches of tryptically digested ICPL-labeled proteins, we recommend to select **ArgC** as enzyme instead of trypsin. ArgC, which cuts C-terminal of Arginine, is the better choice, because trypsin does not cut behind the ICPL-modified lysines. Choosing trypsin as enzyme would mean to increase the number of allowed missed cleavages significantly (Figure 1-10).

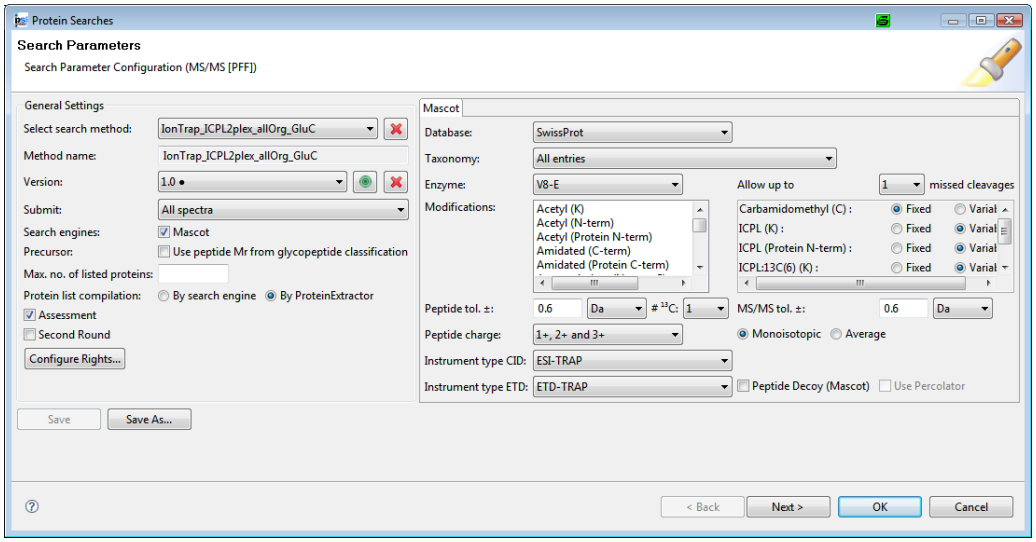

Figure 1-9 **Protein search method IonTrap_ICPL2plex_allOrg_GluC: peptide search parameters.**

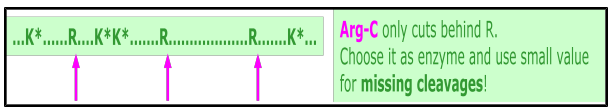

Figure 1-10 **Blocked lysine cleavage sites for ICPL-labeled proteins, where Trypsin cuts only C-terminal of Arginine as indicated by the pink arrows.**

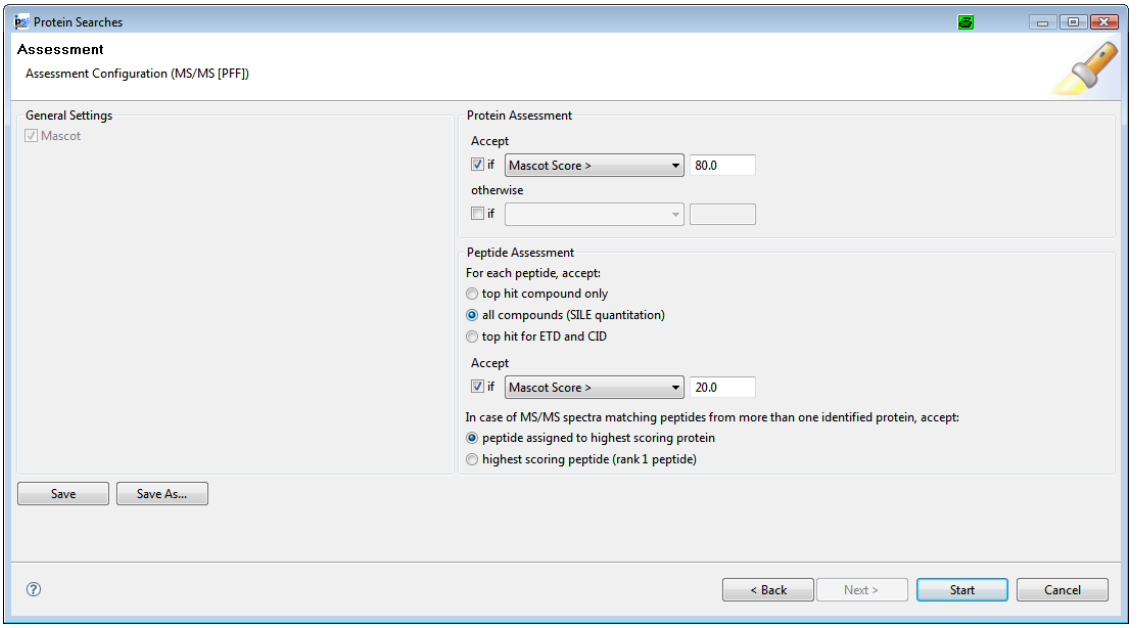

Figure 1-11 Protein search method IonTrap_ICPL2plex_allOrg_GluC: assessment parameters.

1.8.2 Quantitation on one Database Search Result

When the search result is available, the quantitation is started via the button **Quantitation** on the Info tab of the Search Result. WARP-LC is started and asks for an appropriate WARP-LC method, that contains the parameters for the quantitation.

For **non-isobaric labels**, the integration of the MS intensities over a chromatographic peak in an Extracted Ion Chromatogram (EIC) is done only for SILE pairs with at least one identified partner. Thus, WARP-LC triggers DataAnalysis to generate the required EICs and calculate the integrated intensities. WARP-LC calculates the SILE ratios for all pairs and sends the results back to ProteinScape, where the protein ratios are calculated (see section 1.9.2).

For **isobaric labels**, the intensities of the reporter ions are used for quantitation.

1.8.3 Quantitation in 2D Separation Workflows

In ProteinScape, a SILE Quantitation can be started not only for a Search Result of one LC-MS/MS dataset. It can also be started from a ProteinExtractor Search Result that was

generated by compiling proteins from a number of single ProteinExtractor Search Results (Figure 1-12). As an example, Figure 1-13 shows the quantitation result of a 1D gel with 21 bands.

- Each band was excised. The proteins were digested, and each digest was analyzed in an LC-MS/MS run
- 21 LC-MS/MS peaklists were transferred to ProteinScape and stored under 21 bands of a 1D SDS PAGE gel.
- For each band ProteinScape started a ProteinExtractor search.
- All 21 search results were selected, and an overall protein list was generated (context menu **Result Processing | Protein List Compilation**).
- On the Info tab of this new combined Search Result the quantitation was started.
- ProteinScape calculated the quantitation ratios for the proteins based on the peptides of all analyses.

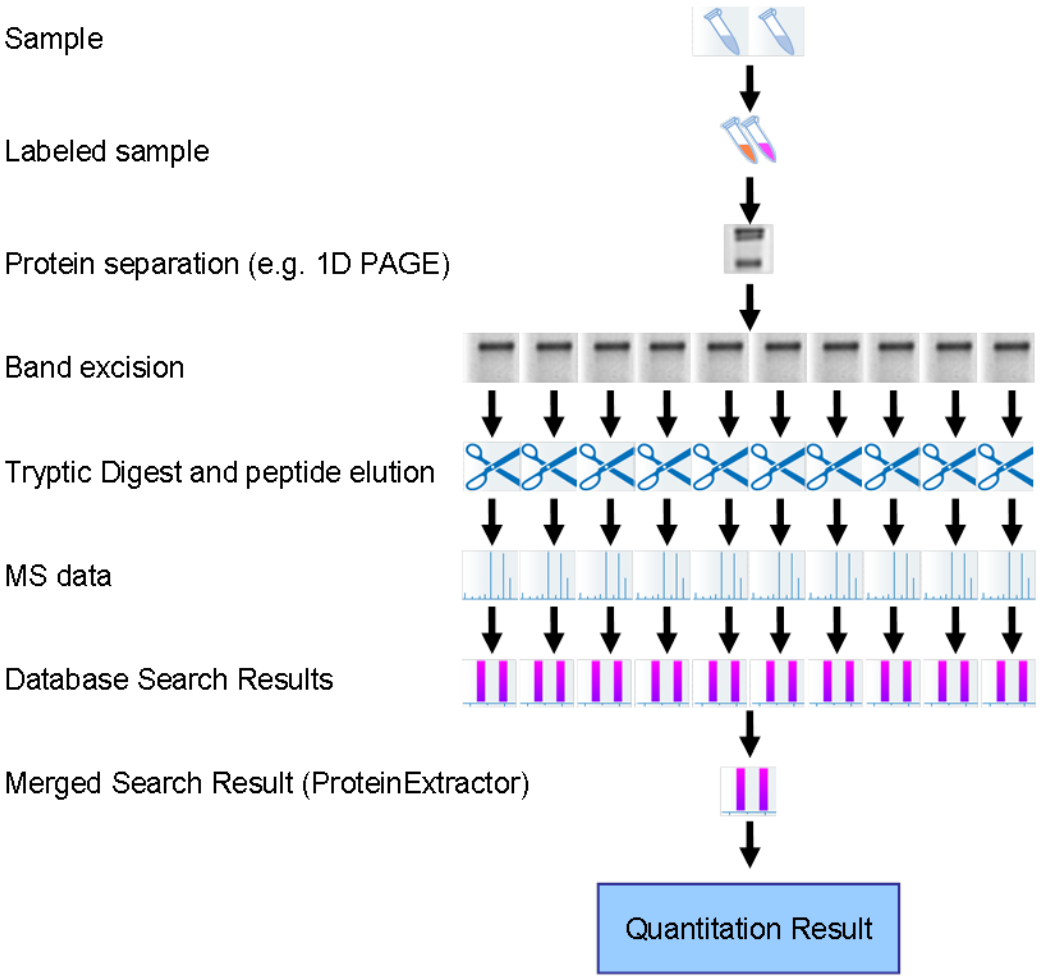

Figure 1-12 **Quantitation of 10 gel bands. This scheme is valid for LC fractions as well.**

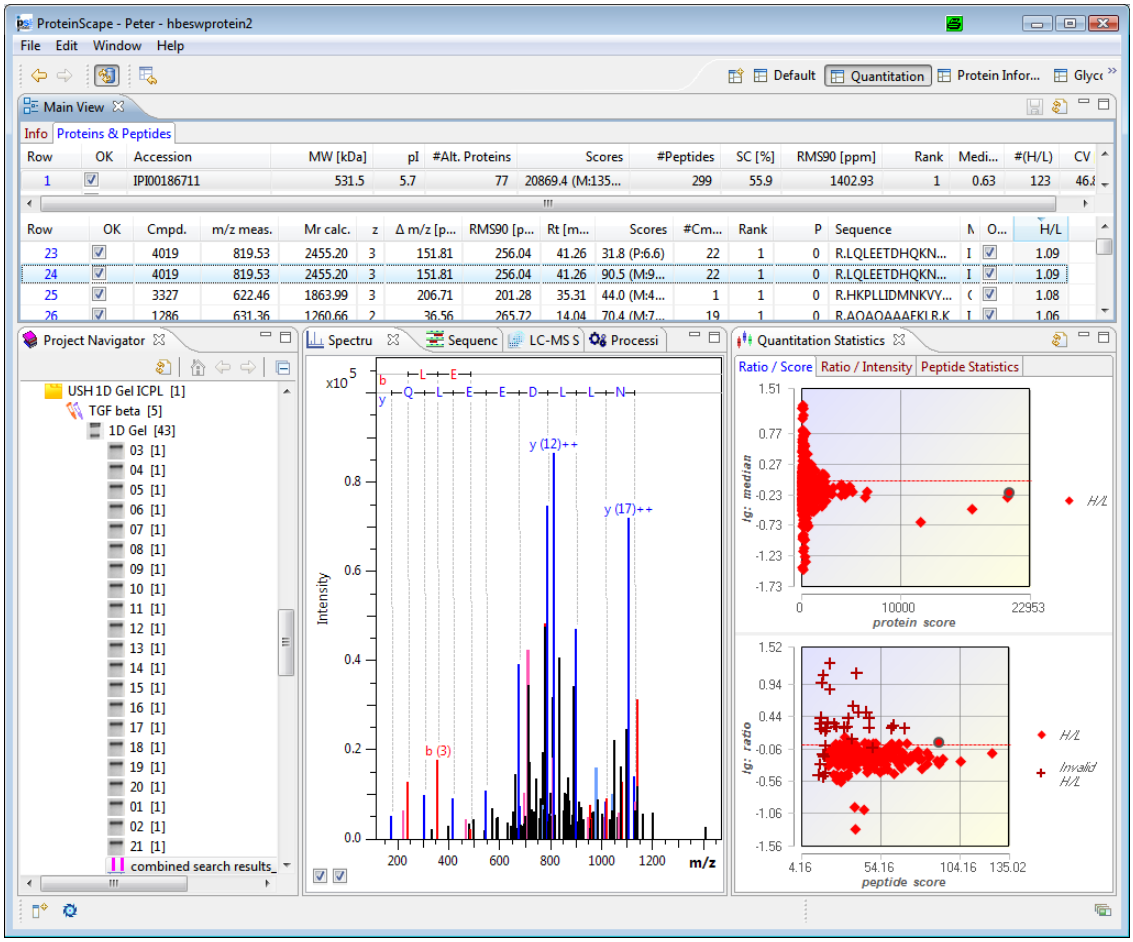

Figure 1-13 **Quantitation perspective with the Quantitation result of an SDS PAGE + LC-MS/MS separation workflow. The Project Navigator shows a Mixed Sample made by mixing two labeled Samples. The Search Result shows the quantitation values for peptides and proteins. For the selected peptide, the annotated MS/MS spectrum is shown. The Quantitation Statistics plot presents the score ratio plot.**

1.8.4 Result Handling in ProteinScape

As soon as the quantitation is finished, the results are available in the ProteinScape Protein Browser and in the Statistic Viewers.

1.8.4.1 Quantitation Result Table

The Protein Browser displays quantitation results for proteins and peptides in additional columns (Figure 1-13). For peptides, ratios and checkboxes for acceptance of ratios are provided. For proteins additional columns for number of peptide ratios, CV values and median are shown. All peptides with quantitation checkbox ON are considered for quantitation. **Outliers** are recognized automatically and are marked with an asterisk (not shown). **Extreme values**, i.e. peptide ratios outside the dynamic range of quantitation, are marked with an arrow up or down. Neither extreme values nor outliers are included in the protein ratio calculation. An **exclamation mark** next to the Median of the selected protein indicates the presence of an extreme value for at least one peptide.

If the checkmarks in the OK (ratio) column are changed manually, the new protein values (sequence coverage, number of peptides identified, number of peptides quantified, Median, CV) are recalculated automatically. This can be changed by deactivating the **Auto Refresh is ON** button which is located on the top left of the screen. Then, manual refreshes on the Protein & Peptide table is required.

1.8.4.2 Statistic Viewers

For a selected protein, the context menu **Show | Quantitation Statistics** opens a Viewer with three tabs, representing the quantitation result (Figure 1-14).

Ratio/ Score tab (Figure 1-14): this viewer is shown for peptides and proteins. Peptides and proteins with outstanding regulation and high scores can be detected easily.

Ratio/ Intensity tab (Figure 1-15): peptides with high intensity and high regulation stand out in this viewer.

Peptide Statistics tab (Figure 1-16): Here, a **Box Plot** is shown for all peptide ratios for one protein. The colored rectangle marks the **upper and lower Quartile**, and the black line shows the **Median**. The black dot indicates the **arithmetic mean**. The highest and lowest value is marked by the **Whiskers**. Moving the mouse over the rectangle opens a tooltip that shows the respective numbers.

The Box Plot is a useful tool, since the difference between the arithmetic mean and the median of the protein regulation is an indicator for unusual and unexpected peptide regulation distributions. This is because the arithmetic mean is more sensitive to the inclusion of outliers compared to the median.

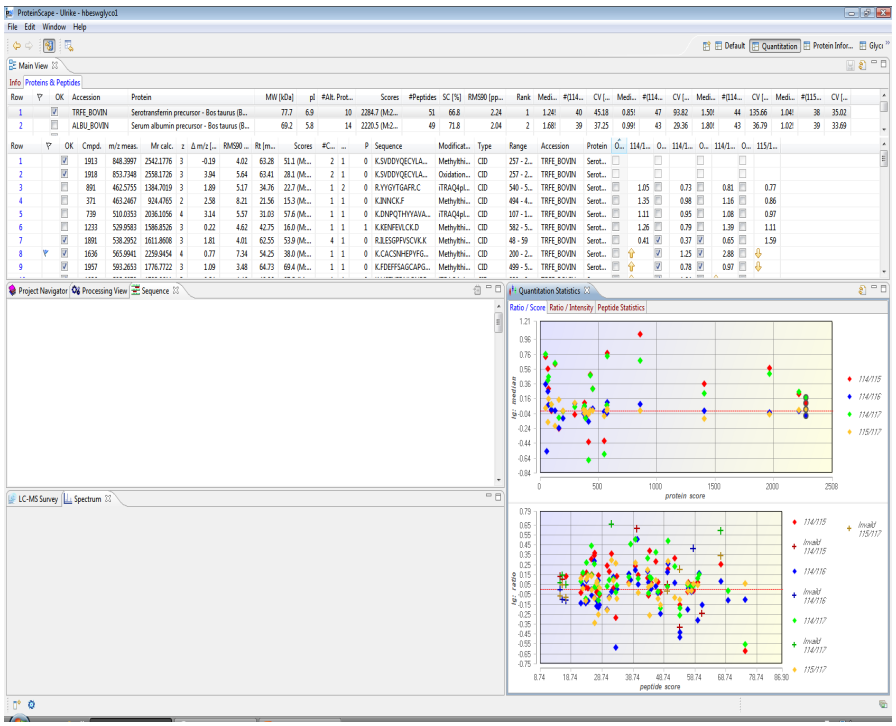

Figure 1-14 Quantitation Statistics Viewer: Ratio/ Score plot

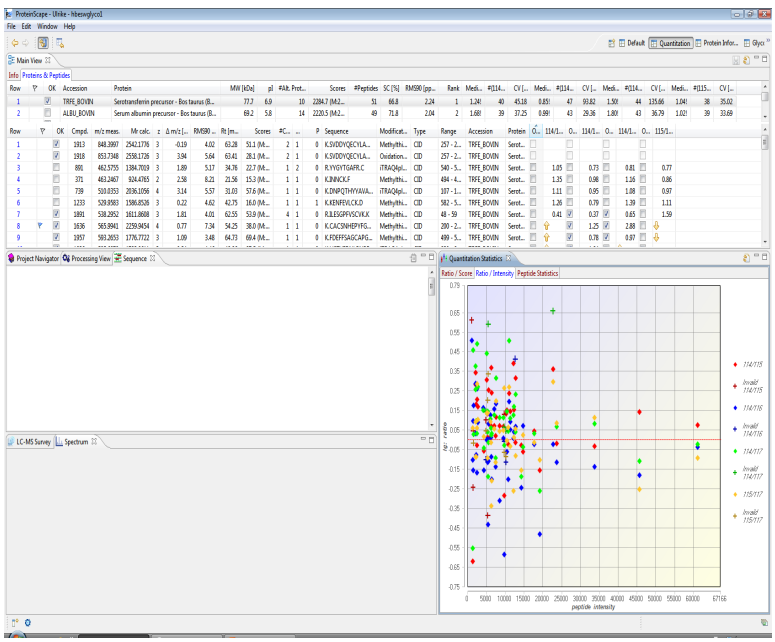

Figure 1-15 Quantitation Statistics Viewer: Ratio/ Intensity plot

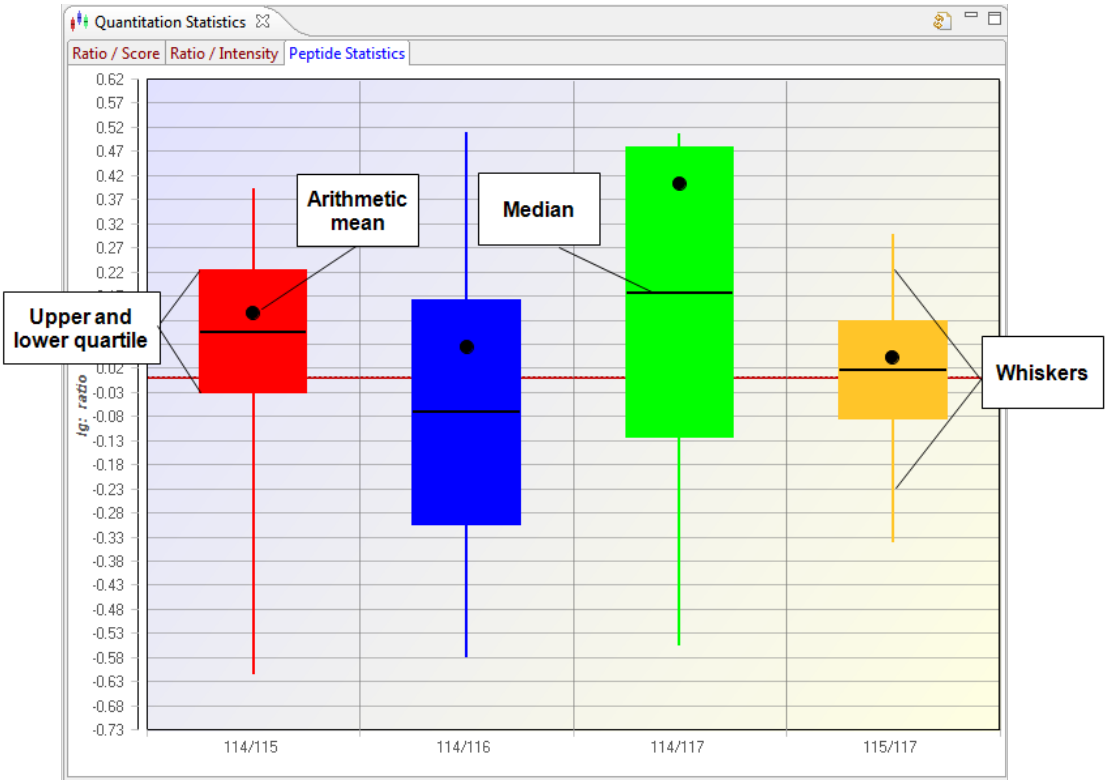

Figure 1-16 Quantitation Statistics Viewer: Box Plot

1.8.4.3 SurveyViewer

For data with non-isobaric labels, it is very helpful to have a close look on the raw data, which can be loaded in the SurveyViewer using the **Load LC-MS raw data** button.

1.8.5 Tools for improving the Quantitation Result

1.8.5.1 Normalize Data

The peptide ratios of unregulated proteins should show a normal distribution about the median value (= 1). However, errors in generation and preparation of samples may lead to a shift of the median. The resulting quantitation errors can be corrected by a normalization step.

In ProteinScape the normalization is performed by dividing peptide ratios by a normalization factor. This value can be the overall median of all peptides or a user-defined denominator.

Click **Normalization** in the **Search Results Info** section of a Search Result's Main View (Figure 1-14). Select **By overall median** to use the overall median of all peptides. Alternatively, clear the **By overall median** check box and type a denominator in the **Factor** field next to the relevant ratio. Clicking **OK** will apply the normalization factor to the entire search result.

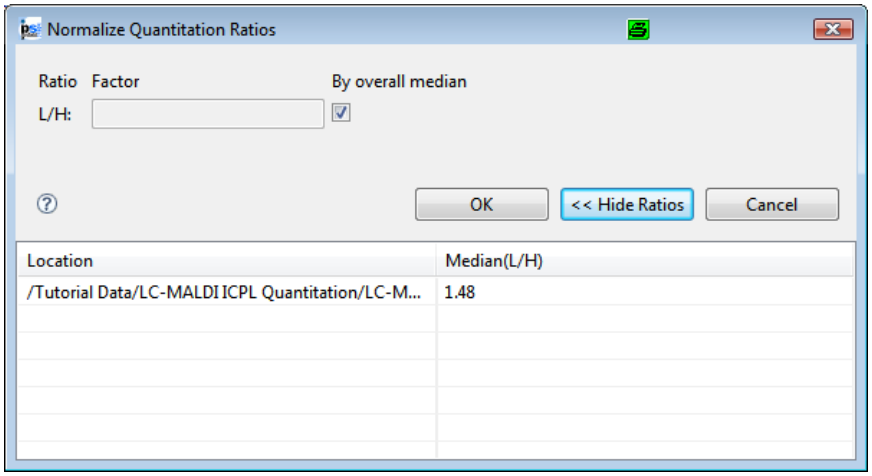

Figure 1-17 Normalize Quantitation Ratio window

1.8.5.2 Uncheck Peptide Ratios

Clicking a check box in the **OK (ratio)** column switches its status between selected not-selected. Only selected ratios are considered for statistical calculation.

If the peptide ratio check marks are changed manually, the new protein table values (sequence coverage, number of peptides identified, number of peptides quantified, median, CV) are recalculated automatically or upon Auto Refresh of the table.

1.8.5.3 Create EICs and change Peptide Ratios

Peptides with unexpected ratios can be selected in the peptide part of the Protein & Peptide table. **Create EICs** is selected from context menu to create EICs from the labelled peptide and its partner(s). Manually, the chromatograms can be integrated, and ratios can

be calculated from the areas or intensities. In ProteinScape the respective columns can be selected in the Peptide table to activate the context menu **Change Quantitation Ratios**.

On the main view of the search result, this change is indicated in the Quantitation Summary section.

1.8.6 Export and Report

The result can be handled in several ways, e.g.:

- The protein list can be exported to Excel.
- All proteins can be selected (context menu) the complete peptide list can be exported.
- On the Search Result Info page, **Show Protein Report** can be activated to export all quantitation relevant data.

1.8.7 Tutorial Data

1.8.7.1 Workflow

Manually import the peaklist `HCT_ICPL_Mix.xml` from `D:\Data\Tutorial Data\WARP-LC\HCT_ICPL_Mix.d` (default installation location) as described in the User's Manual to a Sample or any lower level in ProteinScape. Use the search method `IonTrap_ICPL2plex_allOrg_GluC`. Make sure to use V8-E as enzyme, since the digest has been performed with GluC (V8E) in this experiment. Start the database search, and start quantitation as described in chapter 1.8.2 using the WARP-LC method `WLC_1.3_IonTrap_ICPL2plex_Default.warplcmethod`.

Note When the peaklists (XML) have been moved after data processing, the raw data path in the XML is out of date. This can be corrected in ProteinScape in the field for the Baf/ Yep File, which is located on the MS Data Main View.

1.8.7.2 Analysis of the Quantitation Result

Use the Quantitation Perspective for further analysis of the quantitation results. Details for Perspective handling are described in the ProteinScape User's manual.

This chapter describes the in-detail analysis of one unusual peptide ratio for BSA. One of the peptide ratios of BSA (protein hit # 1) is 1.26, which is unusually high compared to the other BSA peptide ratios. This becomes visible e.g. in the ratio-intensity plot in the

QuantitationStatistics viewer. Upon creating the respective EIC in DA (context menu **Create EICs** on the peptide), smoothing and integration is performed. When having a look on the compound MS/MS spectrum, it becomes evident that the MS signal of the heavy partner at 733.4 is overlaid by another signal. This prevents reasonable quantitation, and the checkmark for quantitation for this peptide should be removed. This reduces the median and the CV value for BSA.

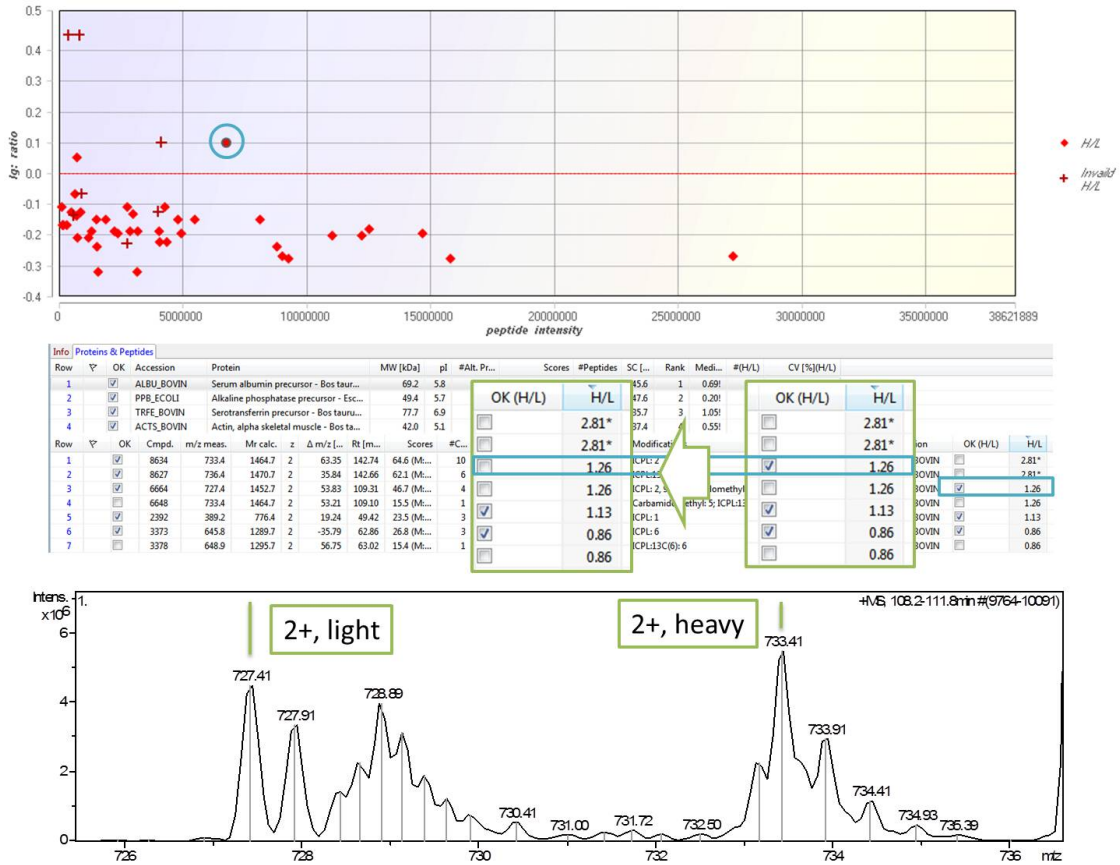

Figure 1-18 **Evaluation of an unusual peptide ratio for BSA, which was detected in the ratio intensity plot. In the spectrum of the integrated compound in DataAnalysis the overlapping signals become evident. In this case, the manual removal of the ratio is recommended.**

1.8.8 Introducing new Labeling Chemistry

For Mascot 2.3, modifications are defined by opening `http://yourmascotservername/mascot/x-cgi/ms-config.exe` in the Internet Explorer and applying the **Add new modification** button. Details are described in the Mascot manual. These new modifications can be introduced in ProteinScape. Please contact the Bruker service for support.

1.9 Automatic Workflow

When starting the measurement from the Sample Table, the following steps can be performed automatically:

- Sample separation
- Data acquisition
- Data processing and peak list export (DA script required, see below)
- Peak list transfer to ProteinScape
- Start of protein database search

1.9.1 HyStar

Requirements:

- An adequate DataAnalysis method containing an automation script needs to be selected in the Sample table.
- The Run DataAnalysis script option needs to be ON for each sample, which is supposed to be handled in an automated way.
- For each sample, the Analyte in ProteinScape, where the peaklist should be transferred, needs to be defined via **View | Get ExperimentID**.

Note On the HyStar computer, the PS client is required. We recommend installing the complete ProteinScape 3.0 client.

1.9.2 DataAnalysis

Requirements:

- The script (Figure 1-4) needs to be contained in the DataAnalysis method.

Note In case of a non-isobaric SILE experiment DataAnalysis is used for data processing and for calculation of extracted ion chromatograms after a protein database search. You should avoid running these procedures simultaneously on one computer, e.g. when running multiple LC-MS/MS runs.

1.9.3 PushDaemon for Peak List Transfer to ProteinScape

The Bruker Push Daemon supervises selected folders for new peaklists created by DataAnalysis and triggers the export to ProteinScape. The respective location here is defined prior to the start of the data acquisition in the HyStar Sample table. Further details are described in the ProteinScape User manual.

Requirements:

- For the automatic peaklist transfer to ProteinScape, the PushDaemon has to be turned ON.
- It has to be configured.
- For automatically starting database searches, the search method is connected on the Sample level. Whenever a dataset is exported to a level below this sample, the search is started automatically. Please refer to the ProteinScape User manual for details.

Note On the computer, where the data are located, the PS client is required. We recommend installing the complete ProteinScape 3.0 client.

Appendix A — Customer Familiarization

Required installations:

- ProteinScape 3.0
- DataAnalysis 4.0
- WARP-LC 1.3
- WARP-LC tutorial data

If you have never used DataAnalysis in an automated workflow before, please start DataAnalysis via the start menu.

		PASS	FAIL
A 1	**Quantify tutorial data**		
1.	In ProteinScape, create a project (name it e.g. Familiarization) and a sample (name it e.g. HCT ICPL).		
2.	Manually import the peaklist ProteinAnalysisResults.xml which is contained in the HCT_ICPL_Mix.d folder (chapter (see "Tutorial Data " on page 9)) by activating the new Sample in the ProteinScape Navigation Tree, and selecting **Import MS Data** from the Context menu. The Import dialog is described in the ProteinScape User's Manual.		
3.	Check that the import is finished in the Processing View. Then, activate the MS data in the Navigation Tree, and activate **Protein Search** in the Main View, which opens the Database Search Method dialog. Use the method IonTrap_ICPL2plex_allOrg_GluC. Check the search parameters, especially the V8-E as enzyme, since the digest has been performed with GluC.		
4.	**Start** the database search, and check the progress of the search in the Processing View.		
5.	When the search is finished, check the result, e.g. for ICPL labeled peptides.		

6.	Start **Quantitate** on the Main View. The WARP-LC dialog opens, where the method WLC_1.3_IonTrap_ICPL2plex_Default.WarpLCMethod is selected. The quantitation starts automatically. Since ICPL is a non-isobaric label, DataAnalysis starts creating EICs and integration.
7.	When the quantitation is ready, analyze the results performing the following steps:
8.	Look on peptide and protein ratios and CV values.
9.	Check the QuantitationStatistics view.
10.	Check ratios in the SurveyViewer (with raw data).
11.	Normalize ratios.
12.	Send compounds to DataAnalysis via the context menu **Create EICs**, and perform smoothing and integration.
13.	Print a report.